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Brief Report

Pex14 is the sole component of the peroxisomal translocon that is required for pexophagy

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Pex14 was initially identified as a peroxisomal membrane protein that is involved in docking of the soluble receptor proteins Pex5 and Pex7, which are required for import of PTS1- or PTS2-containing peroxisomal matrix proteins. However, *Hansenula polymorpha* Pex14 is also required for selective degradation of peroxisomes (pexophagy). Previously we showed that Pex1, Pex4, Pex6 and Pex8 are not required for this process. Here we show that also in the absence of various other peroxins, namely Pex2, Pex10, Pex12, Pex13 and Pex17, pexophagy can normally occur. These peroxins are, like Pex14, components of the peroxisomal translocon. Our data confirm that Pex14 is the sole peroxin that has a unique dual function in two apparent opposite processes, namely peroxisome formation and selective degradation.

Introduction

Autophagy is a highly conserved process that is responsible for the recycling of cytoplasmic components by the vacuole/lysosome.[1] Both selective and non-selective autophagic processes have been described (ref. 2). Pexophagy involves the selective degradation of peroxisomes. In the methylotrophic yeast *Hansenula polymorpha*, pexophagy is induced upon a shift of methanol-grown cells to glucose.[3,4] This process, termed macropexophagy, initiates with the recognition and subsequent sequestration of a single peroxisome by multiple membrane layers, followed by fusion of the outer sequestering membrane layer with the vacuole membrane and finally degradation of the entire organelle by vacuolar enzymes.[5]

Besides numerous autophagy-related (*ATG*)[6] genes, also the peroxisomal membrane protein Pex14 was shown to play an essential role in pexophagy, most likely in the initial recognition of the organelle to be degraded by the autophagy machinery.[3,7,8] Initially, Pex14 was identified as a peroxin involved in matrix protein import as it is thought to recruit the PTS1 and PTS2 receptor proteins, Pex5 and Pex7, respectively, to the peroxisomal membrane.[9,10] Later studies identified two other components of the receptor docking complex, Pex13 and Pex17. A second protein complex in the peroxisomal membrane that is involved in matrix protein import is formed by three RING finger proteins, Pex2, Pex10 and Pex12. Both the docking and RING finger complexes can associate to form a super-complex.[11,12] Here we show none of the peroxins of the peroxisomal translocon besides Pex14 are essential for pexophagy.

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Material and Methods

Organisms and growth. The *H. polymorpha* strains used in this study are listed in Table 1. Cells were cultivated at 37°C using either YPD medium (1% yeast extract, 1% peptone, 1% glucose), or mineral medium (as described previously, ref. 13). For analysis of peroxisome degradation, cells were precultivated using mineral medium containing 0.25% (w/v) ammonium sulphate and 0.25% (w/v) glucose as sole nitrogen and carbon sources respectively. Cells were subsequently shifted to 0.05% (w/v) glycerol and 0.5% (w/v) methanol as carbon sources. Exponential glycerol/methanol cultures were shifted to medium containing 0.5% (w/v) glucose to induce pexophagy.[7] When required, media were supplemented with 30 μg/ml leucine or appropriate antibiotics. For growth on plates, 2% agar was added to the media. *Escherichia coli* DH5α was used as host for propagation of plasmids using LB supplemented with appropriate antibiotics at 37°C as described.[14]

Construction of strains. Each of the *pex* strains as well as wild-type and mutant *atg1* were transformed with either SphI-linearized pHIPX4 *N50.PEX3.GFP*[15] or pHIPZ4 *N50.PEX3.GFP*[16] (as described, refs. 14 and 17). producing N50.Pex3.GFP under the control of the alcohol oxidase promoter.

Microscopy. For fluorescence microscopy, 1 ml cell culture was supplemented with 2 μM FM 4-64, incubated for 45 minutes at 37°C and analyzed with a Zeiss Axioskop microscope (Carl Zeiss, Göttingen, Germany). Electron microscopy was performed as described previously (ref. 18). Ultrathin sections of unicryl-embedded cells were used for immunocytochemistry, using polyclonal antiserum against GFP and gold-conjugated goat anti-rabbit antiserum.

Biochemical methods. Cell extracts were prepared as detailed previously (ref. 15). Equal volumes of the cultures were subjected to SDS-polyacrylamide gel electrophoresis, followed by western blot analysis.[19,20] Blots were probed with rabbit polyclonal antiserum against alcohol oxidase, Pex10 or GFP followed by detection using either the Protoblot immunoblotting system (Promega Biotec)

or BM Chemiluminescent Western blotting kit (Roche Molecular Biochemicals, Almere).

Blots were scanned using a densitometer (Biorad GS-710, Hercules, CA, USA) and quantified using Image J (version 1.37); three measurements were performed on each band of three individual experiments per strain.

Results

To investigate whether besides Pex14 other peroxins of the docking or RING finger sub-complexes are required for glucose induced pexophagy, we studied degradation of peroxisome remnants in single *PEX* deletion strains (*pex2, pex12, pex13* and *pex17*). The degradation of peroxisomal membrane remnants was monitored by the analysis of the levels of the peroxisomal membrane protein Pex10, using western blotting and anti-Pex10 antibodies.[3]

Table 1 **Strains used in this study**

Strain	Characteristics	Reference
wild-type	NCYC495 *leu1.1* derivative	25
atg1	*atg1* deletion strain *leu1.1*	22
pex2	*pex2* disruption strain *leu1.1*	21
pex12	*pex12* disruption strain *leu1.1*	26
pex13	*pex13* disruption strain *leu1.1*	Komori M, Osaka, Japan
pex17	*pex17* disruption strain *leu1.1*	Komori M, Osaka, Japan
WT N50.Pex3.GFP	wild-type:: pHIPX4.*N50.PEX3.GFP*	15
atg1 N50.Pex3.GFP	atg1:: pHIPZ4.*N50.PEX3.GFP leu1.1*	This study
pex2 N50.Pex3.GFP	pex2 pHIPX4.*N50.PEX3.GFP*	This study
pex10 N50.Pex3.GFP	pex10:: pHIPZ4.*N50.PEX3.GFP*	This study
pex12 N50.Pex3.GFP	pex12:: pHIPX4.*N50.PEX3.GFP*	This study
pex13 N50.Pex3.GFP	pex13:: pHIPX4.*N50.PEX3.GFP*	This study
pex17 N50.Pex3.GFP	pex17:: pHIPX4.*N50.PEX3.GFP*	This study

Development of various peroxisome remnants[21] was induced by growing the cells on a mixture of glycerol and methanol, followed by a shift of these cells to glucose-excess conditions. The levels of the peroxisomal membrane protein Pex10 gradually decreased in all mutants similar to the wild-type controls, although the kinetics of degradation may slightly vary (as shown in Fig. 1A). This is exemplified by the data from *pex17* cells that showed a relatively slow decrease of Pex10. As a negative control, *atg1* cells were analyzed in which pexophagy is blocked.[22] In these cells the Pex10 levels did not significantly decrease upon a shift of the cells to glucose medium.

To confirm degradation of the organelles in the vacuole, we also used an artificial marker protein, consisting of the first 50 N-terminal amino acids of Pex3 fused to enhanced Green Fluorescent Protein (eGFP; N_{50}.Pex3.GFP). The first 50 N-terminal residues of Pex3 contain targeting information for the peroxisomal membrane and serve as specific anchor to mark the peroxisomal membrane with GFP. However, this short peptide of Pex3 is not functional in peroxisome biogenesis or degradation.[15]

Electron microscopy showed that the hybrid protein also normally sorted to the peroxisome remnants (shown for *pex13*, Fig. 1B). In fluorescence microscopy, these remnant structures are visualized as single fluorescent spots per cell.[23] We also introduced the *N_{50}.PEX3.GFP* expression cassette in two control strains, namely in wild-type and *atg1* cells.

Western blot analysis revealed that upon a shift of N_{50}.Pex3.GFP-producing wild-type cells from glycerol/methanol to glucose, the levels of the hybrid protein gradually decreased (Fig. 1C). The decrease of the protein levels of peroxisomal alcohol oxidase (AO) indicated that synthesis of N_{50}.Pex3.GFP did not influence degradation of a homologous marker protein. Degradation of peroxisomal proteins was paralleled by the appearance of green fluorescence in the vacuoles as shown in Figure 1D, in contrast to full length Pex3 which is removed from the peroxisomal membrane before uptake of the organelle by the vacuole.[24] In *H. polymorpha atg1* cells producing N_{50}.Pex3.GFP, however, N_{50}.Pex3.GFP and AO levels did not decrease (Fig. 1C) and GFP-fluorescence was not observed in the vacuoles (Fig. 1D).

In addition to *pex2, pex12, pex13* and *pex17* we now also studied the fate of peroxisomal ghosts in *pex10* cells. Each of the *pex* mutants producing N_{50}.Pex3.GFP was pre-cultivated in media containing glycerol/methanol and exposed to excess glucose conditions. Western blot analysis revealed that upon the shift N_{50}.Pex3.GFP levels decreased in all mutant strains, similar to the wild-type control (Fig. 2A, shown for *pex10* cells; Fig. 2B, quantification). Also by this method, the kinetics of degradation was slowest in *pex17* cells. Fluorescence microscopy revealed that in all cases green fluorescence appeared in the vacuole (Fig. 2C; only shown for *pex13* cells). To confirm that in *pex17* cells the peroxisomal remnants were indeed subject to pexophagy, we analyzed *pex17* cells by electron microscopy upon a shift of glycerol/methanol grown cells to glucose. Shortly after induction of pexophagy, additional membranes were formed around the peroxisomal remnants, a typical feature of the initial stages of pexophagy (Fig. 3).[4] Taking together these and earlier data, we conclude that of all *H. polymorpha* peroxins available yet, solely Pex14 is involved in selective peroxisome degradation.

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References

1. Levine B. Cell biology: Autophagy and Cancer. Nature 2007; 446:745-7.
2. Meijer WH, van der Klei IJ, Veenhuis M, Kiel JAKW. *ATG* genes involved in non-selective autophagy are conserved from yeast to man, but the selective Cvt and pexophagy pathways also require organism-specific genes. Autophagy 2007; 3:106-16.
3. Veenhuis M, Komori M, Salomons F, Hilbrands RE, Hut H, Baerends RJS, Kiel JAKW, van der Klei IJ. Peroxisome remnants in peroxisome deficient mutants of the yeast *Hansenula polymorpha*. FEBS Letters 1996; 383:114-8.
4. Sakai Y, Oku M, van der Klei IJ, Kiel JAKW. Pexophagy: Autophagic degradation of peroxisomes. Biochim Biophys Acta 2006; 1763:1767-75.
5. Dunn Jr WA, Cregg JM, Kiel JAKW, van der Klei IJ, Oku M, Sakai Y, Sibirny AA, Stasyk OV, Veenhuis M. The selective autophagy of peroxisomes. Autophagy 2005; 1:75-83.
6. Klionsky DJ, Cregg JM, Dunn WA, Emr SD, Sakai Y, Sandoval IV, Sibirny A, Subramani S, Thumm M, Veenhuis M, Ohsumi Y. A unified nomenclature for yeast autophagy-related genes. Dev Cell 2003; 5:539-45.
7. Bellu AR, Komori M, van der Klei IJ, Kiel JAKW, Veenhuis M. Peroxisome biogenesis and selective degradation converge at Pex14p. J Biol Chem 2001; 276:44570-74.
8. De Vries B, Todde V, Stevens P, Salomons F, van der Klei IJ, Veenhuis M. Pex14p is not required for N-starvation induced microautophagy and in catalytic amounts for macropexophagy in *Hansenula polymorpha*. Autophagy 2006; 2:183-8.

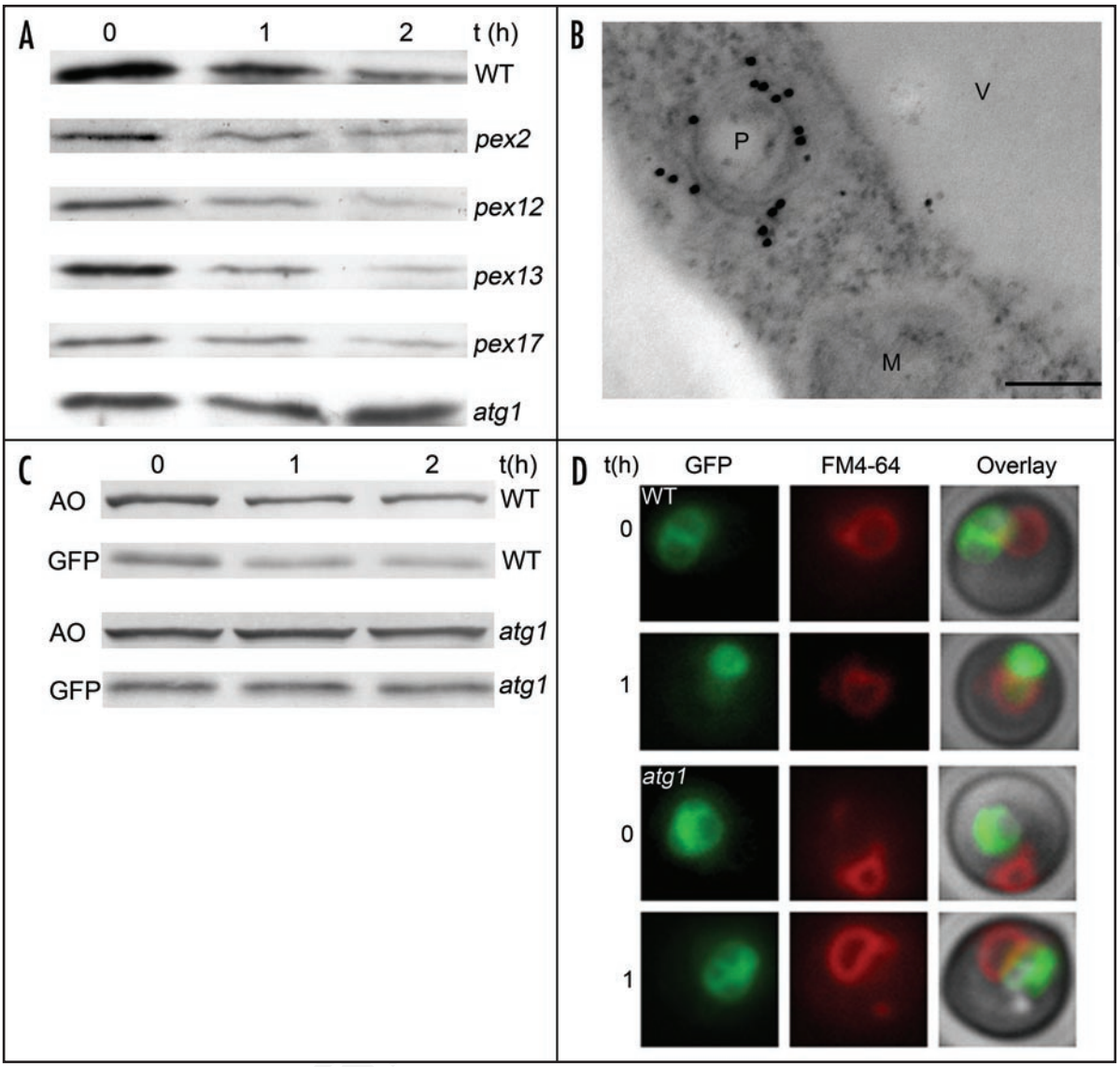

Figure 1. (A) Western blot analysis of Pex10 levels after a shift of glycerol/methanol grown cells into fresh glucose medium. As in wild-type (WT) control cells, the Pex10 levels decreased in *pex2, pex12, pex13* and *pex17* cells in the initial hours of cultivation. In *atg1* control cells the Pex10 levels did not significantly decrease. Samples were taken at the indicated time points after the shift; equal volumes of the culture were loaded per lane. The blots were decorated using α-Pex10-antibodies. (B). Immunocytochemistry to show that N_{50}.Pex3.GFP is exclusively sorted to the peroxisome membrane remnants in *pex13* cells. Ultrathin sections were incubated with α-GFP antibodies and gold-conjugated goat-anti rabbit antiserum (P, peroxisome remnant; V, vacuole; M, mitochondrion. The bar represents 0.2 µm). (C) Western blots showing the decrease of GFP or alcohol oxidase (AO) protein levels upon a shift of WT and *atg1* control cells from glycerol/methanol medium into glucose. Both strains produced N_{50}.Pex3.GFP. Blots of WT cells showed a decrease in AO and GFP protein, which was not observed in *atg1* controls. Equal volumes of the culture were loaded per lane. The blots were decorated using α-AO or α-GFP antibodies, respectively. (D) Fluorescence microscopy of WT and *atg1* cells producing N_{50}.Pex3.GFP. Cells grown on glycerol/methanol medium contain a cluster of 2-3 GFP-marked peroxisomes located adjacent to the vacuole (marked by FM 4-64). In WT cells shifted to glucose medium vacuolar GFP was detected, suggesting that peroxisome degradation had initiated. This vacuolar GFP was never observed in the *atg1* control strain.

9. Komori M, Rasmussen SW, Kiel JAKW, Baerends RJS, Cregg JM, van der Klei IJ, Veenhuis M. The *Hansenula polymorpha PEX14* gene encodes a novel peroxisomal membrane protein essential for peroxisome biogenesis. EMBO J 1997; 16:44-53.
10. Albertini M, Rehling P, Erdmann R, Girzalsky W, Kiel JA, Veenhuis M, Kunau WH. Pex14p, a peroxisomal membrane protein binding both receptors of the two PTS-dependent import pathways. Cell 1997; 89:83-92.
11. Hazra PP, Suriapranata I, Snyder WB, Subramani S. Peroxisome remnants in pex3Δ cells and the requirement of Pex3p for interactions between the peroxisomal docking and translocation complex. Traffic 2002; 3:560-74.
12. Agne B, Meindl NM, Niederhoff K, Einwächter H, Rehling P, Sickmann A, Meyer HE, Girzalsky W, Kunau WH. Pex8p: An intraperoxisomal organizer of the peroxisomal import machinery. Mol Cell 2003; 11:635-46.
13. Van Dijken JP, Otto R, Harder W. Growth of *Hansenula polymorpha* in a methanol-limited chemostat: Physiological responses due to the involvement of methanol oxidase as a key enzyme in methanol metabolism. Arch Microbiol 1976; 111:137-144.
14. Sambrook J, Fritsch EF, Maniatis T. Molecular Cloning; A Laboratory Manual. New York: Cold Spring Harbor Laboratory Press, 1989.
15. Baerends RJS, Faber KN, Kram AM, Kiel JAKW, van der Klei IJ, Veenhuis M. A stretch of positively charged amino acids at the N terminus of *Hansenula polymorpha* Pex3p is involved in incorporation of the protein into the peroxisomal membrane. J Biol Chem 2000; 275:9986-95.
16. Otzen M, Krikken AM, Ozimek PZ, Kurbatova E, Nagotu S, Veenhuis M, van der Klei IJ. In the yeast *Hansenula polymorpha*, peroxisome formation from the ER is independent of Pex19p, but involves the function of p24 proteins. FEMS Yeast Res 2006; 6:1157-1166.
17. Faber KN, Haima P, Harder W, Veenhuis M, AB G. Highly-efficient electrotransformation of the yeast *Hansenula polymorpha*. Curr Genet 1994; 25:305-310.

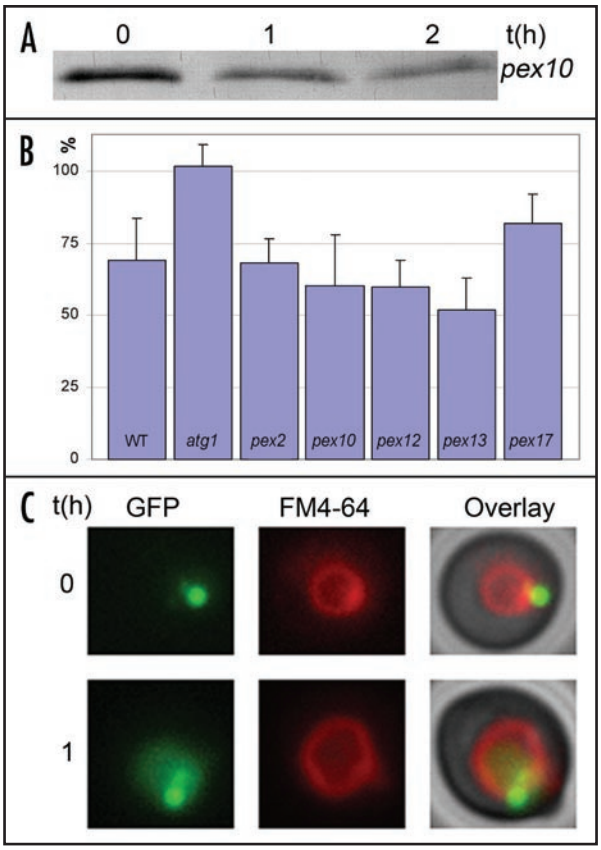

Figure 2. (A) Western blot analysis showing the decrease in N_{50}.Pex3.GFP upon a shift of *pex10* cells from glycerol/methanol to glucose media. Blot is decorated with α-GFP antibodies. (B) Quantification of N_{50}.Pex3.GFP levels in each strain, depicted here as the residual amount of N_{50}.Pex3.GFP protein levels 2 hours after the shift, adjusting the initial amount at T = 0 to 100%. (C) Fluorescence microscopy of *pex13* cells producing N_{50}.Pex3.GFP. The cells contain a single fluorescent spot located adjacent to the vacuole (marked by FM 4-64). After 1 hour of cultivation on glucose, vacuolar GFP is evident, indicating degradation of (part of) these structures.

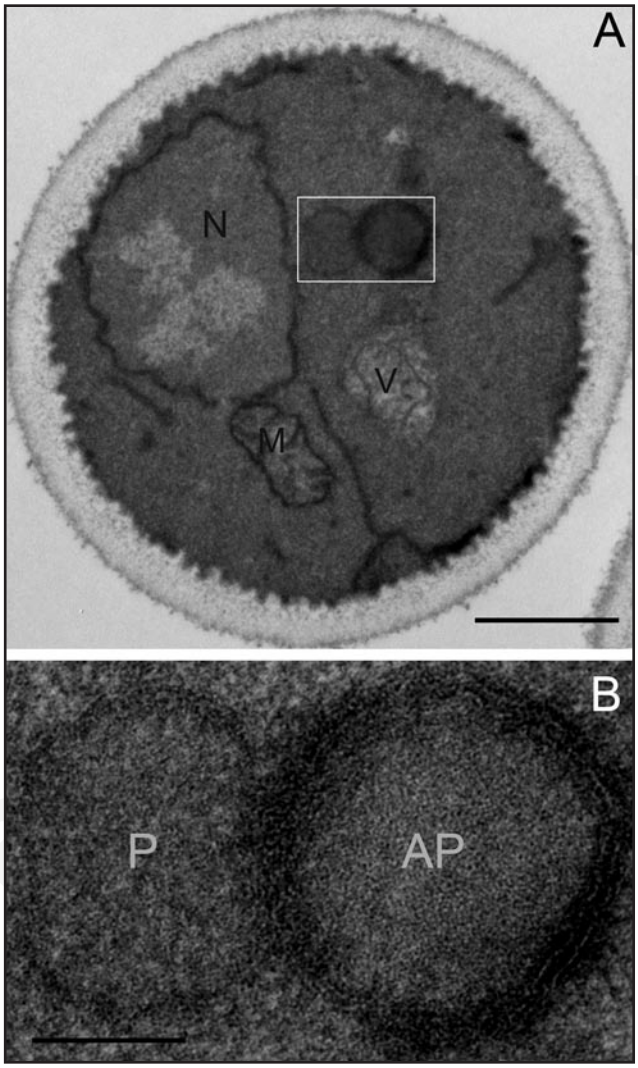

Figure 3. (A) Sequestration of a peroxisomal remnant (by an autophagosome) as observed in *pex17* cells 30 minutes after the shift from glycerol/methanol to glucose. The organelle is sequestered by multiple membrane layers. (B) A higher magnification of the part indicated in (A) is shown. M, Mitochondrion; N, Nucleus; V, Vacuole; P, Peroxisome; AP, Autophagosome. The bar represents 0.5 μm in (A) and 0.1 μm in (B).

18. Waterham HR, Titorenko VI, Haima P, Cregg JM, Harder W, Veenhuis M. The *Hansenula polymorpha PER1* gene is essential for peroxisome biogenesis and encodes a peroxisomal matrix protein with both carboxy- and amino-terminal targeting signals. J Cell Biol 1994; 127:737-49.
19. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970; 227:680-685.
20. Kyhse-Andersen J. Electroblotting of multiple gels: A simple apparatus without buffer tank for rapid transfer of proteins from polyacrylamide to nitrocellulose. Biochem Biophys Methods 1984; 10:203-9.
21. Koek A, Komori M, Veenhuis M, van der Klei IJ. A comparative study of peroxisomal structures in *Hansenula polymorpha pex* mutants. FEMS Yeast Res 2007, (in Press).
22. Komduur JA, Veenhuis M, Kiel JA. The *Hansenula polymorpha PDD7* gene is essential for macropexophagy and microautophagy. FEMS Yeast Res 2003; 3:27-34.
23. Faber KN, Haan GJ, Baerends RJS, Kram AM, Veenhuis M. Normal peroxisome development from vesicles induced by truncated *Hansenula polymorpha* Pex3p. J Biol Chem 2002; 277:11026-33.
24. Bellu AR, Salomons FA, Kiel JA, Veenhuis M, van der Klei IJ. Removal of Pex3p is an important initial stage in selective peroxisome degradation in *Hansenula polymorpha*. J Biol Chem 2002; 277:42875-80.
25. Gleeson MAG, Sudbery PE. Genetic analysis in the methylotrophic yeast *Hansenula polymorpha*. Yeast 1988; 4:293-303.
26. Van Dijk R, Faber KN, Hammond AT, Glick BS, Veenhuis M, Kiel JA. Tagging Hansenula polymorpha genes by random integration of linear DNA fragments (RALF). Mol Genet Genomics 2001; 266:646-656.